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13. ABSTRACT *(Maximum 200 Words)*

In this proposal, we present an innovative approach, tyrosine kinase display, to rapidly identify tyrosine kinases differentially expressed in prostate carcinomas. Our hypothesis is that tyrosine kinases play central roles to the growth and progression of prostate carcinomas. The innovative PCR-based approach developed here requires only restriction digestion and gel electrophoresis, and allows all or nearly all expressed tyrosine kinases to be directly "read" from the gel. It is rapid, quantitative, and insensitive to RNA degradation. When fully developed, it is expected to have wide-applications in both basic and clinical settings. In the past year (May, 1999 to Apr. 2000) we have made the significant progress, notably 1). We have established comprehensive tyrosine kinase profiles for all five widely used prostate cancer cell lines, as well as those of immortalized prostate epithelial cells and the normal primary cultures of prostate stromal and epithelial cells; 2). We have identified several kinases differentially expressed in prostate cancer cells, in androgen-dependent as well as -independent cells. These are potential prognostic markers and intervention targets; and 3). We have identified a new kinase whose expression is induced by DHT and which may mediate signals channeled by androgen receptor. Modulation of this kinase activity may restore androgen sensitivity or block androgen independent growth.

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INTRODUCTION:

Tyrsoine kinases, while a minor class of protein kinases, represent a major class of oncogenes. They are involved in the growth and metastasis of prostate cancer cells (for examples, 1-3) and play key roles in tumor sensitivity to radiation and chemical-induced apoptosis. They are valuable prognostic markers and important targets for intervention (2,4). Kinase inhibitors have recently shown tremendous efficacies and promises in the treatment of human cancers (for reviews see 7 and 8; 9,10). Thus, there is a need to identify the tyrosine kinases expressed in a cancer cell, especially the differentially expressed ones. It has been estimated that there are about 1000 to 2000 protein kinases encoded by the human genome and about 100 of them (i.e., 10%) are tyrosine kinases (6). At present, there are 95 human tyrosine kinases in the GenBank. In a given cell at a given stage, 30 to 50 of them are expressed, a number that is large enough to give tissue or tumor specific characteristics, but small enough to be determined by a simple screen. The present proposal describes an innovative and effective means to display expressed tyrosine kinases of a given prostate cancer cell type, using a single RT-PCR reaction and analyzed by a single gel. Aberrantly expressed or novel tyrosine kinases can be readily identified. There are two major tasks of this proposal:

1. To develop a complete tyrosine kinase display of prostate carcinomas.
2. To identify tyrosine kinases expressed in responses to hormones, drugs and extracellular stimuli.

BODY OF PROGRESS REPORT (May, 1999 to Apr. 2000)

Task 1: To develop a comprehensive tyrosine kinase display of prostate cancer cells.

Tyrosine kinase display of prostate cancer cells We have made significant progress toward this aim. A comprehensive, if not exhaustive, analysis of the tyrosine kinase content of 5 prostate cancer cell lines, LNCaP, CWR22R, DU145, PC3 and TsuPr1 has been done. MLCSV40 was used as a control for normal prostate epithelial cells, so were PrSC (prostate stromal cells) and PrEC (protate epithelial cells), normal primary cultures purchase from Clonetics. RNA samples from all eight cell types were reverse-transcribed and primed by the degenerate primers as previously described (5). The sense primer was labeled with radioisotope by polynucleotide kinase reaction. Each PCR product is thus labeled at the 5'end. The Radiolabeled RT-PCR products were purified from an agarose gel and subject to restriction enzyme digestions, followed by fractionation in a sequencing gel. One example is shown in the Fig. 1, where restriction enzyme MwoI was used ; as can be seen in the figure, all prostate cells display similar, but not identical pattern. (These patterns are quite different from those of the breast cancer cells run in parallel). Importantly, because of the sequence database developed in this year, the sizes of the 5' fragments (i.e., from the labeled 5'end to the first MowI cleavage site) reveal the identities of the tyrosine kinases. The identities of these kinases are listed on the left. CSK (c-src kinase), RON (sea-like kinase), FGFR4 (fibroblast growth factor receptor 4), GC kinase (germinal center kinase) and JAK3 (Jannus kinase 3) can be readily identified. Others with double, triple or multiple designations indicate that the named kinases have identical cleavage pattern and require t more enzyme digestions to solve the ambiguity (see below). However, inspection of the digestion pattern already reveals a number of interesting aspects. First, the only two androgen dependent prostate cell lines in this list are LNCaP and CWR22R

(12-15), which display a pattern more similar to each other than the other androgen-independent, more aggressive cell lines, DU145 and PC3. For instance, FGFR4 is expressed in DU145 and PC3 , but not in LNCaP and CWR22R. Second, compared to normal prostate cells, PrSC and PrEC, the band corresponding to ATK and Mer seems to be expressed at a higher level in the cancer cells. Further digestion analysis concludes that it is Mer (also called Nyk, a N-CAM related tyrosine kinase), but not ATK which is expressed and contributes to the band. This suggests that Mer/ Nyk may be a kinase that is overexpressed in cancer cells. Nyk/mer is a kinase originally cloned by the PI's lab (16, 18) and indepedently Dr. Earp's lab at UNC, Chapel Hill (17). We showed previously that Nyk has a high transforming potential (16), consistent with its being involved in prostate cancer progression. The bands migrating at the top of the gel are not due to incomplete digestions, but correspond to undigested kinases which do not have an MwoI site within this 170bp region of the amplicons. As shown before, this approach is highly quantitative due to the low thermal cycle number needed for the reaction. Phosphoimage analysis of this data file provide an estimate of the expression of these kinases.

Identification of differentially expressed kinases A second sample given here is digestions with HINF I, which also reveals more similarity between LNCaP and CWR22 vs. DU145 and PC3 (Fig. 2). ErbB3 is highly expressed in androgen dependent lines LNCaP and CWR22, but much less in DU145 and PC3. By contrast, Axl kinase is suppressed in LNCaP and CWR22, but not in DU145 and PC3. These data, taken together raise the possibility that some of the kinases such as Axl, RON and FGFR4 may be involved in androgen independence, whereas ErbB3 may be involved in differentiation phenotype (as both DU145 and PC3 are more undefferentiated). This is consistent with the our previous results that treatment of LNCaP by neuregulin or heregulin , the ligand for erbB3 induces cell spreading, stress-fiber formation and the acquisition of a more epithelial-differentiation phenotype (19). In collaboration with Ming-Chie Hung's lab, the tyrosine kinase display approach was used to study kinases that are involved in breast cancer progression. Interestingly, a kinase that is expressed in cancer cells resistant to apoptosis, but suppressed in E1A transfected cells which undergoes apoptosis turns out to be Axl (11). These results taken together suggests that Axl may be involved in the protection of hormone-sensitive cancer cells from hormone-withdraw induced death. Without Axl, the cancer cells (LNCaP, CWR22 and E1A treated breast cancer cells) remain highly sensitive to hormone. Conversely, overexpression of Axl may be one reason why these cells become hormone independent. Further pursuit of the relationship of Axl and hormone independence seems to be warranted. In addition, perhaps non-coincidentally, Mer/Nyk, the kinase found overexpressed in prostate cancer but not normal cells (see above) is a relative of Axl. We also found that the third member of this family of receptor tyrosine kinase, Sky, is also overexpressed in prostate cancer cells. One of the objectives in the future is to study the roles and functions of this family of kinases in prostate cancer progression.

Comprehensive kinase profiles of prostate cancer cells A total of 25 restriction enzymes were used to digest RNA samples from the five prostate cancer cell lines, one normal prostate epithelial cell line (immortalized by SV40LT) and primary cultures of prostate stromal cells and epithelial cells. These 25 enzymes are AciI, AluI, HinfI, HpaII, MnlI, MseI, NciI, RsaI, BstUI, BstYI, BstNI, MwoI, Bsp1286, BsaHI, Cac8I, StyI, AccI, AvaII, HaeIII, ApaL1+HaeII, BslI, BsrI, TaqI, PstI+BglII, XbaI+BseRI. They were chosen so that all tyrosine kianses in the data

band will yield unique signature fragments at least twice. Some of the digestions using a combination of enzymes was just for the purpose of distingusing closely related kinases. With this extensive analysis, we have obtained profiles for all these cell lines. Below is a list of all kinases expressed in these cell lines, each with variable intensity in different cell lines. They are: ABL, ARG, AXL, BRK, CLK1, CLK3, CLK4, CSK, DDR1, DDR2, EGFR, ERBB2, ERB B3, EMK, EPHA1,EPHA2, EPHB2, EPHB3, EPHB4, FER, FGFR2, FGFR3, FGFR4, FYN, JAK1, JAK3, TYK2, KFC-B, KFC-C, LCK, MAK-B, MKK4, MLK2, MLK3, NYK, PDGFRA, PDGFRB, PLK1, RON, SKY, TRK-C, VEGFR3. This is by far the most comprehensive profile of tyrosine kianses in prostate cancer cells. We believe this represent at least 80% of tyrosine kianses expressed and are in the process of completing profiling the remaining 20% and measure the relative intensities of all these kinases.

Toward automation of the procedures Using Cy3 and Cy5-dCTP (Amersham) during the reverse-transcription-polymerase-chain reaction, we have done a test run on using capillary electrophoresis to study the digestion profile, in an effort to automate the display. The results are clear and promising. However, we also
found that the quantitation is not as good as phosphoimaging of the autoradiogram. The intensity range using fluorescence is relatively small. Thus this approach will allow us to identify the types of kinases in a sample with high throughput set up, but the quantitation or the expression level measurement will be compromised.

Task 2: To Identify tyrosine kinases expressed in responses to hormones, drugs and extracellular stimuli.

Androgen treatment With androgen treatment, we have identified a novel kinase AIK (androgen induced kinase), the human homolog of rat MAK (male associated kinase) which is activated about 6 fold at the transcriptional level (20, 23). A full length cDNA was cloned and used to study its expression level. The expression of this kinase is restricted to testis and hence the term, male-associated kinase. Normal prostate tissues do not express this kinase at a high level nor do the cancer tissues. However, after androgen treatment, the expression level of this kinase becomes higher. To confirm this observation, a real-time PCR was used to quantify the level of MAK in LNCaP cell line after androgen treatment. Real time PCR is a recently developed approach which permits precise quantitation of the RNA concentration by monitoring the reaction kinetics of the PCR, thus making PCR quantitative (21). Our lab has a Biorad, iCycler instrument, which allows us to conduct such an analysis. Briefly, specific primers which can amplify MAK transcripts are used to generate RT-PCR products. The thermocycle at which the reaction reaches 50% yields the rate of reaction, which is proportional to the initial concentration of the transcript. Different concentrations of DHT (synthetic androgen) were used and the results are summarized in Fig. 3. The induction is at the highest level when 10 nm DHT is used; higher or lower concentrations all have less potency. There are two conclusions drawn from this result. First, it is clear from this quantitative analysis that MAK expression is induced by DHT, confirming the tyrosine kinase display results, and attests to the sensitivity of the display approach (the expression level of MAK is so very low , beyond the detection by standard Northern analysis). Second, the dose response curve of MAK induction parallels that of the growth response, indicating that MAK may be involved in mediating DHT induced growth of

LNCaP cells. To our knowledge this is the first protein kinase which is induced by androgen at the transcription level. Together with the recent finding that androgen signals induces MAP kinase (22), this result strengthens the hypothesis that androgen receptor signaling may involve kinase and phosphorylation cascade. It will be of great interest to study the kinases involved in such as cascade. It is conceivable that deregulation of some of these downstream kinases may account for androgen-independent growth. Inhibition for these kinases could either terminate the constitutive signals or restores the hormon-sensitivity. Thus, there is a great need to understand the nature and the mechanisms of action of MAK. From the structural data, we know this kinase is in the dual-kinase family (i.e., sequences are similar to kinases which phosphorylate both serine/threonine and tyrosine residues). The catalytic domain carries motifs analogous to both MAPK and cdc2. We also know now that there are two other related kinases in the same family. We designate them as MAK-B and MAK-C. MAK-B is also testis specific, but does not seem to be induced transcriptionally by androgen; MAK-C is ubiquitously present in many tissues. We will be interested in pursuing the functions of MAK and its regulation by androgen.

Growth induction: In the original proposal, we also wished to test whether growth conditions would alter the tyrosine kinase expression profiles. We did a careful analysis of the cell density effect on the expression profiles. There are two reasons to conduct this type of experiment; first, when the cells reach certain density, they usually slow down their growth and the tyrosine kinase profile may identify kinases involved in this process. Second, the increased density facilitates cell-cell communication and we may be able to identify kinases which respond to cell-adhesion signals. Tsu-Pr1 cell line was used to test how cell-cell communication may affect the kinase expression. Cells grew to 30%, 70%, 100% or >100% (i.e., piling up) confluency were used. An example is given on the right panel of Fig. 1., where RNA isolated from Tsu-Pr1 cells grown to different density was subject to RT-PCR and restriction digestion. The kinases and their expression level were identified as before. We found that the expression of most of the kinases was not affected by cell density. There are however, a few interesting exceptions. The expressions of EGFR and FGFR3 decrease, as cell density increase, whereas those of RON and JAK3 increase. It would be of interest to define the nature of the cell-cell communication that mediate this transcriptional regulation and to understand the roles of the above kinases in regulating growth and senescence. This project is ongoing and will be extended to the next grant period.

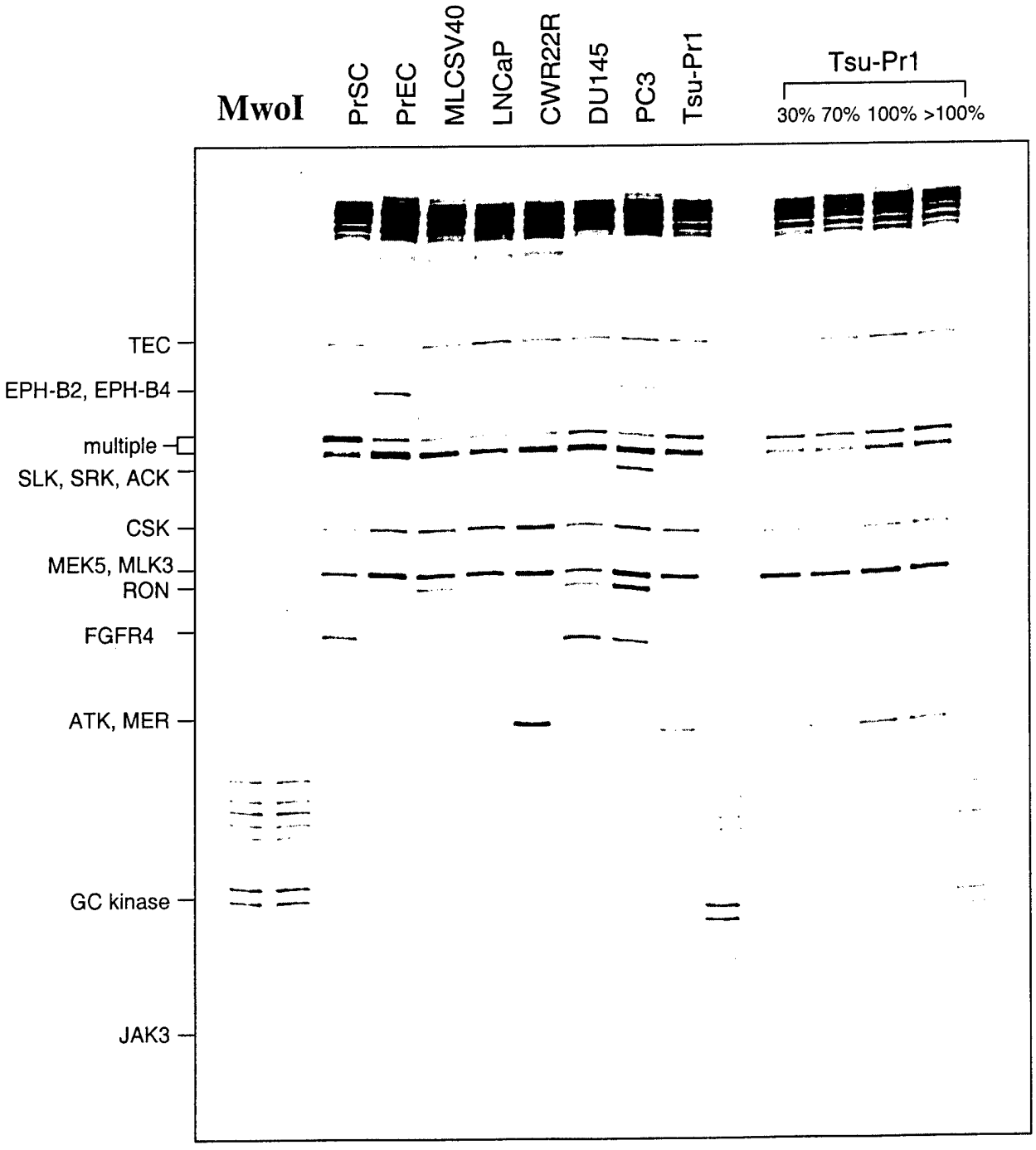


Figure 1. Tyrosine kinase display of prostate cancer cells (I). The procedure and the samples are outlined in the text and the enzyme used for digestion is MwoI. On the left panel, different cell lines cultured under identical conditions were used for RNA extractions. On the right, Tsu-Pr1 cell line grown to different densities (percentages of confluency are shown on the top of the gel) were used for kinase display. The names of the kinases identified are listed on the left. Multiple means that these bands could come from more than one kinase, because identical cleavage site occurring in multiple kinase amplicons.

Tyrosine Kinases in Prostate Cell Lines

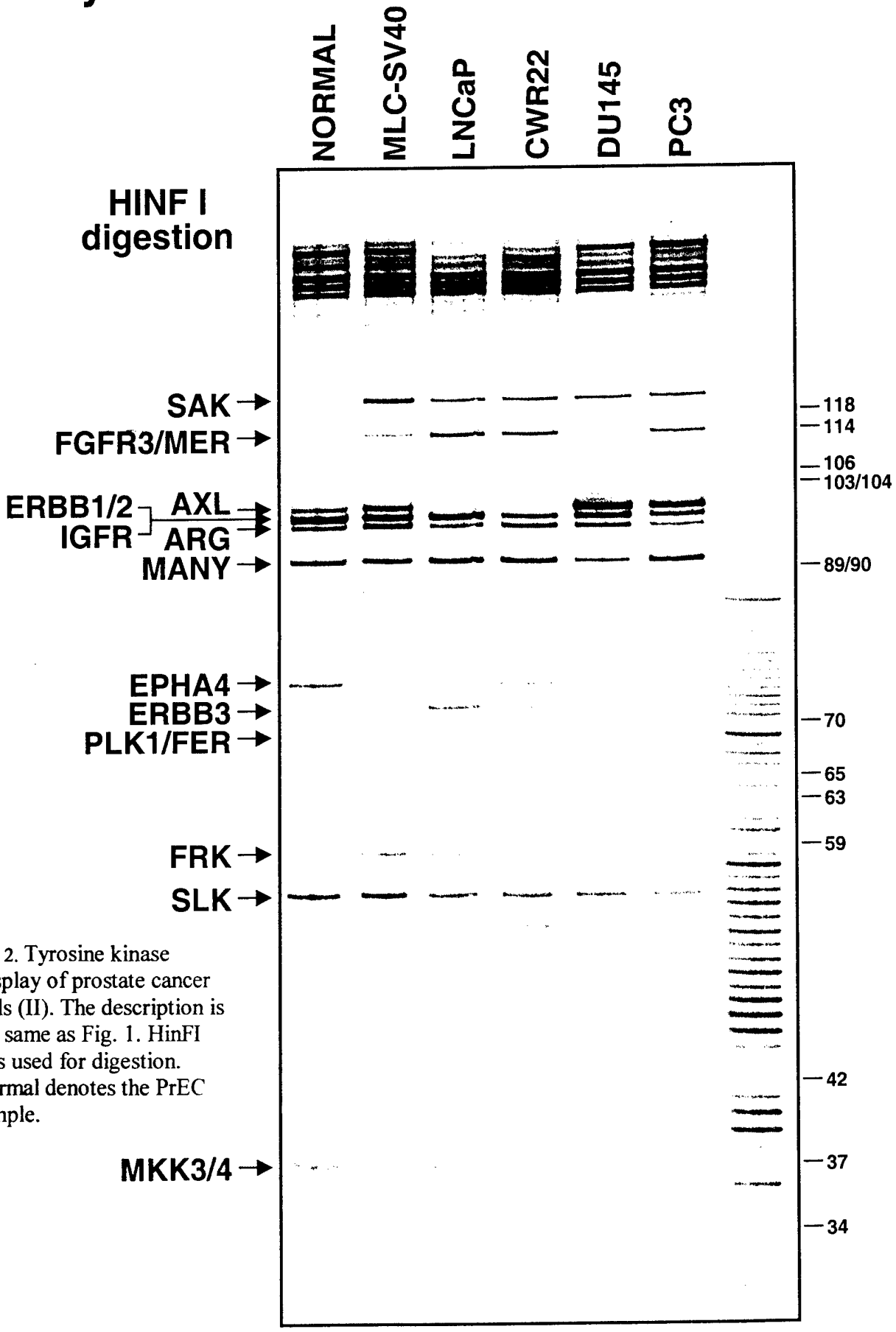
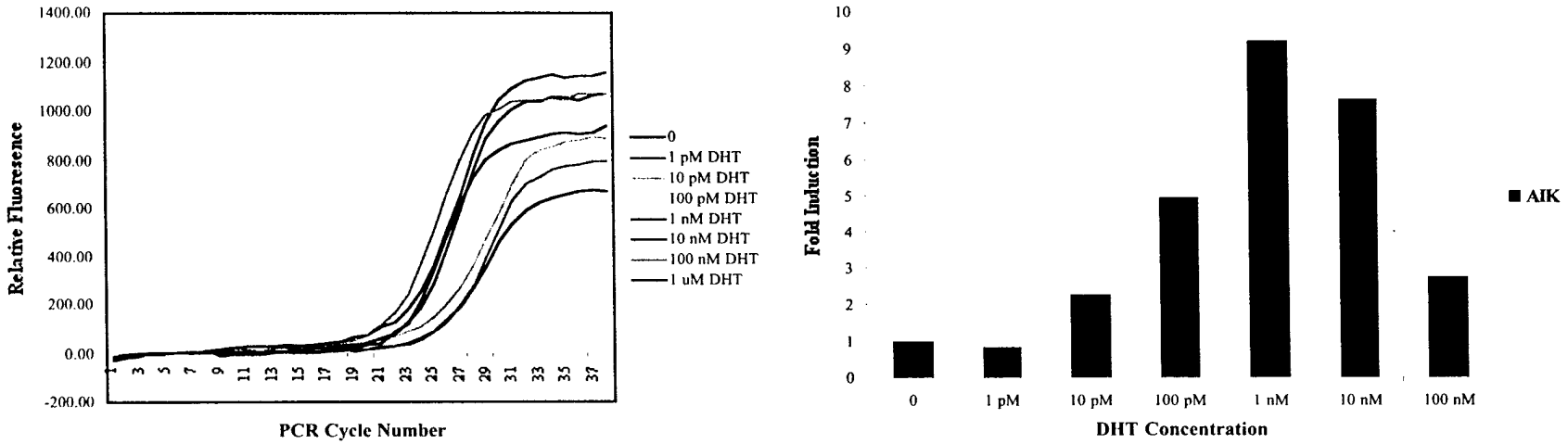


Figure 2. Tyrosine kinase Display of prostate cancer cells (II). The description is the same as Fig. 1. HinFI was used for digestion. Normal denotes the PrEC sample.

Androgen Induced AIK mRNA Expression Detected by Real Time PCR



A B

Figure 3. Real-time RT-PCR kinetic study of AIK expression in response to DHT treatment. A 433 base pair fragment of AIK cDNA was amplified from reverse transcribed RNA samples of LNCaP cells with or without DHT treatment. Relative fluorescence versus cycle number is plotted for each sample to permit simple visualization of the exponential phase of amplification (A). Results were normalized by comparison with the amplification of a 304 base pair β-actin cDNA. Induction folds of AIK expression level of DHT treated samples over untreated ones are shown (B).

KEY RESEARCH ACCOMPLISHMENTS

- Established comprehensive tyrosine kinase profiles for five prostate cancer cell lines (LNCaP, CWR22R, DU145, PC3, Tsu-Pr1), one immortalized prostate epithelial cells (MCSV40), and two primary prostate cultures of stromal and epithelial cells.
- Identified several kinases whose expressions vary among androgen-dependent and – independent cell lines.
- Discovery of a new kinase whose expression is induced by DHT, an androgen analogue.

REPORTABLE OUTCOMES

- One abstract: Xia, L., Robinson, D., Chen , H.C., Ma, A.H., and Kung, H.J., (2000) AIK, a novel androgen-inducible kinase, identified by tyrosine kinase display of prostate cancer cells. Proceedings of AACR March 2000, 41: 252 (appendix)
- One manuscript: "Tyrosine kinases and cellular signaling in prostate cancer" by Hsing-Jien Kung, Clifford G. Tepper, and Ralph W. deVere White. To be published in Prostate Cancer in the 21th Century, ed. by Chung L.W.K. Human Press , in press, (2000) (appendix)

CONCLUSIONS

The present study provides for the first time comprehensive tyrosine kinase profiles for prostate cancers. Since tyrosine kinases are master switches controlling a variety of cellular responses including growth, apoptosis, differentiation, migration, metastasis, chemo- and radio-sensitivity, knowledge about the kinases involved opens avenues to 1). Understand the complex signal pathways; 2). Design strategies for modulating the behavior of cancer cells; 3) Sensitize cancer cells toward chemo- and radio therapies; 4) Develop inhibitors for kinases or associated signal molecules to inhibit cancer cell growth. And 5) Develop agents that may restore hormone-dependence or eliminate hormone-independent cells. In addition, based on our profile data, we have identified several tyrosine kinases which are differentially expressed in prostate cancer cells, in hormon-dependent prostate cancer cells and in prostate epithelial cells. Most remarkably, we have identified a new kinase that is induced by androgen. These kinases are potential prognostic markers and intervention targets for prostate cancer. Following the original objectives proposed in the application, we will extend the analyses to ascertain that these kinases can be used profitably as markers in all or a subset of prostate cancers. In addition, attention will be focused on elucidating the signal pathways associated with these kinases, in the hope to define their roles in prostate cancer growth, metastasis and hormone-independence.

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APPENDICES:

- Appendix I: Xia, L., Robinson, D., Chen , H.C., Ma, A.H., and Kung, H.J., (2000) AIK, a novel androgen-inducible kinase, identified by tyrosine kinase display of prostate cancer cells. Proceedings of AACR March 2000, 41: 252

- Appendix II: "Tyrosine kinases and cellular signaling in prostate cancer" by Hsing-Jien Kung, Clifford G. Tepper, and Ralph W. deVere White. To be published in Prostate Cancer in the 21th Century, ed. by Chung L.W.K. Human Press , in press, (2000)

Appendix I

Presented at American Association for Cancer Research (AACR) 91st Annual Meeting (April 1-5, 2000, San Francisco, CA) ABSTRACT #1606

AIK, a Novel Androgen-Inducible Kinase, Identified by Tyrosine Kinase Display of Prostate Cancer Cells

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Our laboratory is interested in studying kinases and signal transduction pathways involved in prostate growth. We have developed an effective tyrosine kinase display method that allows us to describe all or nearly all tyrosine kinases using a single gel with a single RT-PCR reaction. The complete tyrosine kinase profiles of several widely used prostate cancer cell lines were obtained. This method, with its exquisite sensitivity, also permits the identification of kinases differentially expressed in prostate cancer cells, treated with or without hormone. We report here the identification of a novel, androgen-inducible kinase (AIK), which is modulated by androgen at the transcriptional level. Northern blot analysis revealed that AIK is expressed highly specifically in testis but very low in other normal tissues examined. This male associated kinase contains sequence motifs related to both CDK and MAPK family, and intriguingly, is localized in the nucleus. A tantalizing hypothesis is that AIK may be a downstream kinase, targeted by androgen action and serves to transmit androgen signal. Experiments are underway to define the signal pathways that AIK may be involved.

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TYROSINE KINASES AND CELLULAR SIGNALING IN PROSTATE CANCER[1]

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TYROSINE KINASES AND PROSTATE CANCER

Introduction

There is very strong evidence that tyrosine kinases are involved in the growth and metastasis of prostate cancer (for examples, (66,154,167)). Tyrosine kinases also play key roles in modulating tumor sensitivity to radiation- and chemical-induced apoptosis. There is thus hope that they may play an important role in the response of metastatic prostate cancer to hormonal intervention as well as to other chemotherapeutic approaches (79). Their potential importance as targets for intervention is underlined by the FDA approval of the HER2/Neu-directed therapy, Herceptin, for breast cancer therapy and the present clinical trials investigating its effectiveness for prostate cancer (142). Presently, because of screening, 80 percent of prostate cancers are found while still localized to the gland. One of the major issues is if we could determine which cancers were not going to metastasize, treatment could be given on an individual basis. Presently, prostate specific antigen (PSA) and tumor grade are the best markers we have. While being generally good clinical indicators, they lack specificity for the individual patient. There are a number of indications that tyrosine kinases may be valuable as prognostic markers in these situations (66,154,167).

It has been estimated that there are about 1000 to 2000 protein kinases in the human genome with 100 to 200 of them (i.e., 10%) being tyrosine kinases (60). At present, there are 85 human tyrosine kinases identified in the GenBank database and based on the relatively slow rate of discovery in the past few years, one hundred is a better approximation to the total number of tyrosine kinases encoded by the human genome. In a given cell at a given stage, it is reasonable to assume that there are 30 to 50 tyrosine kinases expressed, a number large enough to provide characteristic tissue-specific patterns, but small enough to be identified in a simple screening. The hope for tyrosine kinases as prognostic markers rests with the fact that the identification of a stage-specific expression pattern will be identified in prostate cancer cells while they remain localized to the gland.

A Tyrosine kinase profile of prostate cancer

In an effort to identify one or more novel biomarker, an effective tyrosine kinase display approach was developed to identify all or nearly all tyrosine kinases expressed in prostate cancer using a single RT-PCR reaction and visualized in a single polyacrylamide gel. The approach takes advantage of common invariable motifs present in the catalytic domain of the great majority of tyrosine kinases (for instance DFG and DVW motifs in subdomains VII and IX, respectively). Degenerate primers based on reverse translation of these highly conserved sequence motifs are used to generate RT-PCR products of tyrosine kinases and the resulting amplicons can be sequenced by traditional means or better yet, subjected to restriction enzyme digestions such that the resulting fragments of different sizes reflect individual kinases. In the latter approach, the identities of the tyrosine kinases can be "read" directly from the gel, saving the time-consuming steps of cloning and sequencing. The band intensity corresponds well to the level of expression of a given kinase. When samples from normal and tumor tissues are compared, overexpressed tyrosine kinases can be readily identified. The first comprehensive tyrosine kinase profile was constructed from an androgen sensitive, prostate specific antigen (PSA)-releasing prostate cancer xenograft CWR22. Table 1 summarizes the data derived from the display approach as well as direct sequencing of the amplicons (120). There are 20 receptor-tyrosine kinases and 12 non-receptor tyrosine kinases. Among the receptor kinases, three (ErbB1, 2, and 3) come from the epidermal growth factor receptor (EGFR) family and four (EphA1, A2, A4 and B4) from the Eph family. In addition to the Eph family of kinases, there are several cell adhesion molecule-related receptor kinases expressed in this CaP xenograft : Sky and Nyk which carry neural cell adhesion molecule (NCAM)-like domains, the discoidin domain receptor tyrosine kinases Ddr1 and 2, and RET which contains a cadherin domain. The presence of EGFR (ErbB1) (see below), nerve growth factor receptor (NGFR, trkA) (40,50), fibroblast growth factor (FGFR) (for examples, (34,52,127,129)), and insulin-like growth factor 1 (IGFR) (86,114) are consistent with literature reports describing the responses of prostate cancer cells to these ligands. Among the non-receptor tyrosine kinases represented, the src family contains three members (src, yes and lck) and the related src-B family member Frk. The initial profile data also revealed several novel kinases, unknown at the time of discovery, but subsequently cloned: Nyk/Mer, an NCAM-related receptor tyrosine kinase (54,81) and Etk/Bmx, a pleckstrin homology (PH)-domain containing tyrosine kinase (116,146). The former has an elevated expression in CaP, compared to normal prostate epithelilal cells, and the latter is expressed at a higher level in LNCaP than in other cell lines, and is implicated in IL-6 induced neuroendocrine differentiation (see below). It is also noteworthy that trkA, trkC, and RET receptor kinases are expressed in CWR22 and LNCaP. This finding was initially surprising, as these kinases are known to be associated primarily with neuronal tissues, but is consistent with the notion that some of the prostate epithelial cells, especially when escaping hormonal control, have neuroendocrine properties and can be trans-dfferentiated into such a lineage (see below). RET has recently been shown to be overexpressed in high-grade CaP and high grade PIN, but not in low-grade samples (35). This finding suggests that RET may play a significant role in CaP progression and raises the interesting possibility that high grade CaP's are derived directly from high grade PIN. Overall, the CWR22 tyrosine kinase profile described above is typical of all CaP's studied, although there is a greater similarity of the tyrosine kinase profiles between the two androgen-sensitive models, CWR22 and LNCaP, than those of the androgen-insensitive lines.

A number of ligands that transmit signals through receptor tyrosine kinases have been implicated in prostate cancer transformation and progression. This review will focus on the EGF receptor family of kinases and the signals transmitted by these receptors. The involvement of FGFR and NGFR families will be briefly mentioned to serve as reference for further discussion. The literature citations are representative and not meant to be inclusive.

The FGF receptor family of tyrosine kinases

FGF-2 or basic FGF (bFGF), FGF-7 or keratinocyte growth factor (KGF), FGF-8 and FGF-9 are strong mitogens for prostate cells and their production is associated with benign prostatic hyperplasia and CaP development (for examples, (36,53,72,105,123,148)). Both FGF-2 (144) and FGF-7 (162) are provided by the stromal cells under normal conditions, but FGF2 expression in prostate epithelial cells is downregulated by androgen (133). However, during the development of prostate cancer, alternative splicing of the FGFR2 locus leads to a switch in the expression of FGF receptor 2 isoforms from FGFR2(IIIb) to FGFR2(IIIc) with a concomitant shift in affinity from FGF-7 to FGF-2. An important event following this is the up-regulation of FGF2 expression resulting in the establishment of an autocrine loop (162), rendering the cells stromal–independent and androgen-independent (18). In general, the FGF family of growth factors are viewed as progression factors for CaP and the observation that FGF-2 expression is regulated by androgen in prostate epithelial cells suggests that the molecular events leading to hormonal independence may occur at a much earlier stage than presently thought and further implicates it as a critical factor to consider with regards to androgen ablative therapy (133).

The NGF receptor family of tyrosine kinases

NGF has two receptors : the high-affinity receptor, trkA, and the low-affinity gp75NGFR. TrkA is a tyrosine kinase that serves to transduce NGF-induced differentiation and survival signals, whereas gp75NGFR tends to induce apoptosis. In normal prostate, gp75NGFR is expressed in the epithelial cells,

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whereas the ligand, NGFE is expressed in the stroma (111). The expression of gp75NGFR is reduced in prostate CaPs and completely absent in malignant CaP cell lines, Tsu-pr1, DU145, PC3 and LNCaP. Thus, there is an inverse correlation of expression of the gp75NGFR and CaP development. Consistent with its negative role in CaP progression is the finding that artificial expression of gp75NGFR in Tsu-pr1 cell line results in NGF-induced apoptosis (110). In contrast to the low affinity receptor, trkA seems to be expressed in the majority of CaPs and all four CaP cell lines and is a positive growth modulator for CaPs. NGF treatment of these cells stimulate their growth (6) and inhibitors of the NGF/trkA pathway inhibits CaP growth (39,51). It is interesting that while NGF/trkA-induced signaling in neuronal cells results in neuronal differentiation, neuroendocrine differentiation in CaP is induced by agents such as interleukin-6 (IL-6) and forskolin (see below), but not by NGF (6,104). NGF also fails to induce the growth of normal prostate cells and a recent finding suggests that the difference in the biological behaviors between tumor and normal cells cannot be attributed to mutations of trkA (51), but more likely to the presence or absence of gp75NGFR. In two other studies, it was shown that NGF induces invasiveness of DU145 (50) and hormone-independence of Tsu-pr1 (40). Thus, NGF has a dual role in prostate cancer, depending on the repertoire of the receptors present in the cells: trkA behaves as a positive regulator for growth and tumor progression, whereas p75NGFR, an apoptosis inducer.

The ErbB/EGF-receptor family of tyrosine kinases

Among receptor kinases, the ErbB/EGF-receptor family is most frequently implicated in human malignancies. There are four members in this family, namely ErbB1 and ErbB2, ErbB3, and ErbB4 (26,74,112). The majority of prostate carcinomas express ErbB1, ErbB2 and ErbB3, but little or no ErbB4 (120). ErbB1 is the EGF receptor and frequently has been found overexpressed in tumors of epithelial origin. Amplification of ErbB1/EGFR has not been detected in CaP, but overexpression of this receptor is common. In nearly all CaP cell lines or tissues surveyed, an autocrine loop of TGF-Δ/EGF and ErbB1/EGFR exists, thus replacing the requirement for the normal stromal-derived ligand (24,85,96,134,149). Inhibition of ErbB1/EGFR autocrine loop or the kinase activity of the receptor prevents the growth of CaP cells, indicating an essential role of ErbB1/EGFR in their growth (13,120). Interestingly, such an inhibition also affects the actions of IGF-I and protein kinase A (PKA), indicating a general role of ErbB1/EGFR signaling in CaP growth (114).

ErbB2, also called neu (for the rat homologue) or HER2 (*H*uman *E*GF *R*eceptor), is the second member of this family and figures prominently in human malignancies. The ErbB2 gene is amplified and overexpressed in 20 to 30% of primary breast cancers and correlates with poor prognosis. However, a humanized mouse monoclonal antibody against ErbB2/HER2, Herceptin, exploits this feature as a novel molecular target and has shown promise in clinical trials as an anti-breast cancer therapeutic agent, alone or in combination with standard chemotherapeutics (106). Unlike breast carcinoma, genomic amplification of ErbB2 is rarely observed in prostate cancer (14,46,75,89,155), but there are noteworthy exceptions (124-126,136). The expression of both ErbB2 and 3 is either low or undetectable on normal prostate luminal epithelial cells, but is prevalent in prostate adenocarcinoma (83). Accordingly, the expression of ErbB2 is considered to be an early event of CaP transformation (98). The level of ErbB2 expression does not seem to vary significantly among CaP's of different histological grade (58,77), although overexpression of ErbB2 in primary prostatic tissue predicts poor survival (47,94). In addition, elevated serum levels of ErbB2 seem to correlate with progression of the disease status (7) and (95)an association of ErbB2 overexpression with the occurrence of metastatic disease has also been reported (124). Further strong evidence for ErbB2 as an important factor in CaP metastasis was provided experimentally by the demonstration that *in vitro* transfection of rat prostatic epithelial cells with an oncogenic ErbB2 mutant (*i.e. Neu* mutation) resulted in metastatic tumors after orthotopic injection into nude mice (88,168).

ErbB3, the third family member, is a kinase-impaired receptor and requires dimerization with other family members to become an active, signal transducer (59). ErbB3 is expressed in the majority of primary and metastatic CaP's (56,78,98,113,120). The ligand for ErbB3, heregulin (HRG) or neuregulin (NRG), is reported to be expressed in 36% of CaP's analyzed by Leung *et al.* (78) and the autocrine loop of HRG/ErbB3 appears to be associated with less favorable prognosis in advanced CaP's. By contrast, Lyne *et al.* (83) and Grasso *et al* (56) found HRG expression was absent in CaP specimens, three established CaP cell lines (LNCaP, DU145, PC3), and one xenograft (CWR22). HRG, however, is expressed in an immortalized, non-tumorigenic prostate epithelial cell line (56), and is expressed in 100% of stroma and 100% basal epithelial cells, and 58% of luminal cells in normal and benign hyperplastic prostatic tissue (83). The latter studies suggest a down-regulation of HRG and concomitant loss of this autocrine loop during tumor progression, consistent with its growth arrest and differentiation effect on CaP cell lines (see below).

In the ensuing section we will discuss the signaling events elicited by growth factors such as TGF-Δ/EGF and heregulin, and by cytokines such as IL-6, as they pertain to CaP biology. As will be discussed below, depending on the partners the individual receptors associate with, they channel very different signals, with profoundly different biological outcomes.

Tyrosine Kinase Signals through Growth Factor Receptors

EGF/TGF-Δ signals : growth, androgen-independence, survival, and invasion.

Among the peptide growth factors, the action of EGF and TGF-Δ on prostate growth have been most extensively analyzed. There is a preponderance of evidence suggesting the involvement of EGF/TGF-Δ in the growth of prostate epithelial cells, and the autocrine loop of TGF-Δ/EGFR found in virtually all prostate cancer cells plays a significant part in their uncontrolled growth. Addition of EGFR blocking antibody or specific inhibitors of the EGFR kinase (13,169) diminishes the growth of CaP. Inclusion of exogenous EGF and TGF-Δ in growth media further increases the growth rate of the prostate cancer cells and this effect is synergistic with androgen. In the CWR22 xenograft model, the conversion from androgen-sensitive to the relapsed form (CWR22R) correlates with increased expression of TGF-Δ, indicating the TGF-Δ/EGFR autocrine loop may override the requirement for androgen (97). EGF/TGF-Δ stimulation of LNCaP induces tyrosine phosphorylation of EGFR/ErbB1, ErbB2, and ErbB3, with ErbB1 being the strongest. Homo- and hetero-dimer formation of ErbB1/ErbB1, ErbB1/ErbB2 and ErbB1/ErbB3 dimers are all detected. Functionally, however, it appears that the ErbB1/ErbB1 homodimer is the most important. Using a LNCaP cell line where ErbB2 is functionally knocked out by the transfection of a single chain antibody gene directed against ErbB2, it was shown that ErbB2 is dispensable for most of the EGF/TGF-Δ induced growth phenotypes (55). Under these conditions, phosphorylation of ErbB3 is significantly reduced, indicating that ErbB2 mediates ErbB3 phosphorylation. Since only the growth properties of the ErbB2 "knock-out" cells were studied, the role of ErbB2 and ErbB3 in other EGF-induced functions such as migration or survival have yet to be defined.

The intracellular signals are transmitted from membrane-associated tyrosine kinases to serine kinases or lipid kinases, and eventually to transcripitonal factors through phosphorylation cascades. ErbB1/EGFR signals through several pathways in prostate cancer cells : Shc/mitogen-activated protein kinase (MAPK) (19,57,114), phosphatidylinositol 3-kinase (PI3K)/Akt, phospholipase C-ϑ (PLC-ϑ/protein kinase C (PKC), p21-activated kinase (PAK)/Jun N-terminal kinase (JNK), and the signal transducers and activators of transcription (STATs) (56). The prevailing model is that upon ligand binding, homo- or hetero-dimers of the cognate receptors are formed, leading to transphosphorylation and activation of the intrinsic kinase activity. The active kinase is phosphorylated at the tyrosine residues which serve as anchor sites for a number of substrates with *src* homology 2 (SH2) and phosphotyrosine binding (PTB) domains, resulting in the phosphorylation of these substrates. Different substrates define the engagement of different pathways, although there is strong evidence that these pathways are interconnected and tend to modulate one another. A combination of the signal outputs from individual pathways define the eventual phenotypes of the receptor activation. In the following sections, we review what is known about the signals involved in the growth, hormone-independent growth, survival, and motility induced by EGF/TGF-Δ. Other than motility and invasion where DU145 is a better model, the experimental data were principally derived from LNCaP studies. A summary diagram of the various signal transduction pathways is shown in Fig. 1.

The growth signals :

Among the several pathways listed above, MAPK seems to be the most important in channeling the growth signals and the initial transformation of the cells. An early clue that attests to the importance of this pathway for growth

and transformation comes from the fact that both ras and raf which lie upstream in the pathway are potent oncogenes and growth stimulators in a variety of cell types. In prostate cancer cells, mutations of ras or raf are rarely found, yet heightened activation of MAPK is detected in high grade and hormone-independent CaP (19). The TGF-Δ/ErbB autocrine loop found in most of the advanced CaP almost certainly contributes to the persistent activation of MAPK. Other overexpressed tyrosine kinases such as RET and NYK may also play a significant role. These receptors activate MAPK presumably through the well defined Shc/Grb2/SOS/ras/raf/MEK pathway (Fig. 1) (33,43,76,82,108,153). While one knows a fair amount of upstream activators of MAPK, how MAPK drives the growth pathway is still not entirely clear. It has been reported that MAPK is able to phosphorylate c-Myc and Elk. Elk, an Ets-like transcriptional factor is known to augment the expression of the AP-1 complex components fos, jun and jun B. Both the AP-1 complex and c-Myc are known to activate cyclin D thus propelling cells toward S phase. Furthermore, Chen *et al* recently showed that ectopic overexpression of cylcin D stimulates constitutive growth and tumorigencity of LNCaP (21) and Perry *et al* demonstrated that EGF activates cyclin D1 in LNCaP (109), lending support to the above model. The same group further showed EGF-induced activation of Cyclin D1 expression was dependent upon PKC. This also makes sense, since EGF is known to activate PLC-γ which induces intracellular calcium elevations and produces diacylglycerol (DAG), two agonists for PKC, an activator of AP-1 complex. Activation of the AP-1 complex by the MAPK and PKC pathways follows two different phosphorylation cascades that are expected to be synergistic. It seems reasonable to propose that the elevated expression of cyclin D1, due to the combined action of MAPK and PKC pathways, plays an important part in EGF/TGF-Δ induced growth of LNCaP. It is noteworthy that LNCaP has wild type p53 and Rb genes, making the overexpression of cyclin D1 necessary to overcome the actions of these cell cycle gate-keepers. LNCaP however carries a mutant PTEN, which encodes a phosphatase specific for the product of PI3K, phosphatidylinositol 3,4,5-trisphosphate (152). Deficiency of PTEN is thought to let loose the PI3K activity which confers survival through Akt activation (see below). However, at least one recent report implicates PTEN in G1 growth arrest and points to an unrecognized function of Akt in cell cycle progression (119). This indicates that PTEN mutation may also contribute to the aggressive growth properties of LNCaP, although the mechanism is less clear.

The survival signal :

The phosphatidylinositol 3-kinase pathway has recently attracted a great deal of attention in view of its of its diverse effects. PI3K is a lipid kinase which catalyzes reactions to engender 3'-phosphoinositides (Fig. 1). These lipid moieties bind a class of molecules bearing PH domains, which translocate them to the cytoplasmic membrane and often alter their conformation. Akt/protein kinase B (PKB), a serine/threonine kinase involved in anti-apoptosis is one such substrate whose translocation to the membrane allows it to be phosphorylated and activated by 3-phosphoinositide-dependent protein kinase-1 (PDK1), another PH domain-containing serine/threonine kinase activated by PI3K. A direct inhibitory effect on the cell death machinery has been demonstrated by phosphorylation of Bad (32,38) and procaspase-9 by active Akt (16), inactivating their pro-apoptotic functions. Recruitment of the PI3K/Akt pathway can also have a cytoprotective effect via activation of nuclear factor-kappa B (NF-κB) and subsequent up-regulation an anti-apoptotic transcriptional program (103,122). Akt can mediate degradation of the NF-κB inhibitor, IκB, by interacting with, phosphorylating, and activating IκB kinase (IKK). IKK, in turn, phosphorylates IκB and targets it for degradation. NF-κB is consequently liberated and permitted to be translocated to the nucleus. This scheme is postulated to be the molecular basis of PI3K's ability to function as a survival factor. Recent studies by Lin *et al* (80) and Carson and Weber (17), provide direct evidence that PI3K plays a significant role in sustaining survival of LNCaP, based on the observation that PI3K inhibitors, wortmannin and LY294002 induce a high level of LNCaP cell death. These apoptotic effects can be partially rescued by treatment with EGF, which is known to activate PI3K presumably via the activation of ErbB1/ErbB3 heterodimer (80). Interestingly, the Akt activity is not restored (since the PI3K inhibitor is still present) and the authors postulate the existence of Akt-independent survival signals channeled by PI3K. ErbB3 carries multiple PI3K binding sites and is particularly effective in forming a multi-molecular complex with PI3K involving at least 5 additional tyrosine phosphorylated species (Grasso and Kung, unpublished data). It is conceivable that PI3K, having multiple protein-protein interaction domains, may impart signal transduction by serving as an adaptor molecule without invoking lipid kinase activity.

Counteracting PI3K is the lipid phosphatase PTEN/MMAC1, originally discovered as a tumor suppressror gene for a number of cancers including prostate cancers, up to 50% of which are defective in structure or expression of this gene. This phosphatase removes a phosphate from the 3' site of phosphatidyl polyphosphates, particularly phosphatidylinositol 3,4,5-trisphosphate, thereby diminishing the activating signal for Akt. Indeed, prostate cancers such as LNCaP lacking PTEN/MMAC1 have a constitutively high level of activated Akt (157), which may account for this cell line's unusual durability in harsh conditions. LNCaP, while growth arrested, can survive long-term in serum-free and androgen-free conditions.

A new PI3K effector, Etk/Bmx, has recently been identified in prostate cancer cells (116,120). Etk/Bmx is a tyrosine kinase that carries a PH domain at the N-terminus and belongs to the Btk family. Etk is the only member of the Btk family that is expressed in prostate cells such as LNCaP. In a manner similar to Akt, it was shown that Etk is able to protect CaP from thapsigargin or radiation induced apoptosis (161). While the molecular nature of this protective effect remains unclear, this finding demonstrates that there are other potential effectors of PI3K in anti-apoptosis. STAT1,3, and 5 have also been shown to be phosphorylated and activated by Etk/Bmx (130) and their connections to the protective effect of Etk are being scrutinized.

The hormone-independence signals :

LNCaP requires androgen for growth. The autocrine loop of TGF-Δ/EGFR existing in this cell line apparently is not sufficient to override the hormone-dependence; addition of exogenous EGF or IGF-1 however can induce LNCaP growth in the absence of synthetic androgen (29). This suggests that either the EGF signal can activate the androgen receptor (AR) pathway in the absence of androgen (*i.e.* EGF and androgen in the same pathway), or EGF induces an independent growth pathway, obviating the need for androgen (*i.e.* EGF and androgen are in parallel pathways) or both. At least one report showed that the androgenic growth signal requires the interaction between amphiregulin and EGFR (137) suggesting that the EGFR pathway lies down stream of the androgen pathway. In support of this contention are the recent demonstrations that the MAPK pathway is able to activate androgen receptor transcriptional activity in the absence of its ligand (1,28,164). While in the latter studies, the authors utilized overexpressed ErbB2 as a source for MAPK activation, ErbB2 is activated by EGF via heterodimerization with ErbB1, and as described above, EGF is a potent activator of MAPK. Thus it is likely that EGF or TGF-Δ induced androgen-independent growth of LNCaP also follow the same pathways. In support of a role for ErbB2 in the conversion of prostate cancer cells into a hormone-independent state is the finding that prostatic acid phosphatase which diminishes ErbB2 activity restores hormone-sensitivity of a variant LNCaP line refractory to hormone induction (91). If MAPK is the key factor involved in activating AR in the absence of androgen, one would predict MAPK agonists other than ErbB family members should also be able to convert hormone sensitive cells to refractory status. This has yet to be tested. On the other hand, it is equally likely that MAPK is but one of the several pathways activated by ErbB1 or ErbB2 which contribute to AR activation. In the latter case, not all agonists that activate MAPK would induce AR-independence. How does MAPK activate the transcriptional activity of AR? Chen *et al* found that the target for the phosphorylation cascade is AR itself at a site where phosphorylation would strengthen the interaction with cofactors such as ARA50 or ARA70. These factors presumably enhance the DNA binding or transactivation function of the unliganded AR to a similar extent as the liganded AR. There is also evidence that PKA or elevated cAMP level activates AR in the absence of ligand (128). While this is likely due to direct phosphorylation of AR by PKA (128), there are at least two reports indicating a synergy between PKA and ErbB1 in the activation of MAPK in LNCaP (19,114) which may contribute to the androgen-independent activation of AR.

The motility and invasion signals:

EGF is known to induce cell motility, detachment, and invasion of cancer cells. As these processes are dependent upon the cell type and the extracellular matrix used, it is therefore difficult to generalize. For instance, EGF or TGF-Δ seems to have little effect on the cytoskeletal structure, motility, or invasiveness of LNCaP cells, although it promotes chemomigration of Tsu-pr1 cells (118) and motility of DU145 cells (150). In the case of DU145, the activation of PLC-γ seems to be crucial in the migratory properties of the cell,

presumably via the activation of PKC and mobilization of calcium (151). The same authors also showed that disassembly of focal adhesions, a step linked to migration, involves the MAPK pathway (160). In other cell types, growth factor induced cell migration often involves the PI3K pathway and small GTPases such as rac1/rhoA/cdc42 (see below, HRG section).

In addition to cell motility, invasion requires the release of proteinases to digest the extracellular matrix. In prostate cancer, EGF induces the release of matrilysin and urokinase (uPA), two molecules strongly implicated in the invasion process (44,45,68,117,145). The pathway leading to uPA activation involves AP-1 and thus likely includes MAPK and JNK as its effectors (156).

Heregulin (HRG) Signals: growth arrest, cytoskeletal reorganization, and apoptosis

As described above, EGF and TGF-Δ are involved in many aspects of prostate cancer progression by engaging with ErbB family receptors, primarily Erb1 and Erb2. However, ErbB3 and Erb4 are the high affinity receptors for heregulin, a polypeptide ligand that has varied effects on different prostate cancer cell lines (15). HRG was also identified as neuregulin (NRG) and Neu differentiation factor (NDF), among other names, but will be referred to as HRG in this discussion for convenience (65,87,107). Since ErbB4 is not usually expressed in CaP, HRG functions in this cell type primarily via activation of ErbB3/ErbB2 and to a lesser extent, through ErbB3/ErbB1 heterodimers. Under these conditions, an ErbB3/ErbB3 homodimer may also form, but would be unproductive, as ErbB3 is kinase-impaired and requires other kinase-active receptors to transphosphorylate it. Indeed, using a LNCaP cell line that has ErbB2 knocked out by ErbB2 antibody gene-trapping technique, HRG's signal ability is completely abolished (55). Thus, in prostate cancer cells, ErbB2 is a vital component of the HRG signal machinery and the ErbB2/ErbB3 heterodimer is the principal component activated by HRG. Expression analysis of HRG provides an interesting contrast to that of EGF/TGF-Δ: HRG is expressed highly in normal prostate epithelial cells, especially basal cells, and stromal cells, but at low or undetectable levels in prostate cancer cells (56,83). None of the commonly used CaP cell lines express HRG, whereas an immortalized, normal prostate epithelial cell line, MLC-SV40, does. This suggests that NDF may serve a differentiation or anti-proliferative role in prostate cancer cells, as it does in some of the breast cancer cell lines (30,147).

As will be discussed below, some of the intracellular signals, such as MAPK, induced by HRG and by EGF/TGF-Δ are similar, but others are different, and have distinct biological consequences. Some of the notable differences are summarized here (Fig. 2). For instance, after HRG treament, PI3K is assembled into an "activation complex", which can be differentiated from that induced by EGF/TGF-Δ based upon the tyrosine-phosphorylated band patterns present in the complexes. In addition, HRG treament activates p38MAPK and JNK, but not PLC-9 and STATs (56). Akt is activated only moderately over the already high background of constitutive activity in LNCaP.

The growth-arrest signals :

LNCaP grows well in media containing androgen and serum. In the presence of HRG, the growth rate declines, indicating that HRG transmits a dominant growth arrest signal (56,83). Addition of the MEK inhibitor PD98059 does not restore growth of LNCaP treated with HRG nor does the addition of a PI3K inhibitor, LY294002. The latter data however is somewhat difficult to interpret as LY294002 induces apoptosis even in the absence of HRG. How does HRG induce growth arrest? Bacus *et al* (8) demonstrated that HRG induces the expression of p53 and p21WAF1, a CyclinD/CDK2 inhibitor, in LNCaP. Yu et (165) also showed that ErbB2 overexpression upregulates the transcription of p21WAF1. The accumulation of p21 may be one reason why cells go into quiescence. Consistent with this notion is the finding that PDGF treatment of prostate cancer cells results in the induction of p21WAF1 expression, G1 arrest and the sensitization towards radiation-induced apoptosis (71). Additionally, tamoxifen-induced apoptosis of PC3 and DU145 apparently involves the upregulation of p21WAF1 expression (121), as does cell cycle inhibition of DU145 induced by type-I interferon alpha (63). Thus, p21WAF1 may be a common mediator of growth arrest in prostate cancer cells.

The cytoskeletal reorganizaton and detachment signals:

In addition to growth arrest, treatment of LNCaP with HRG induces immediate, cytoskeletal rearrangements with characterized by the formation of filopodia, lamellopodia and stress fibers. A distinct morphological change accompanies this by an alteration in cell shape to a more rounded appearance from the slender shape assumed by typical epithelial cells (55). This is followed by detachment of cells from the plate. Specific inhibitor experiments delineated that this process depends on PI3K rather than MAPK. The involvement of PI3K in shaping the cytoskeletal structure has been well documented in other systems and is thought to engage rac/rho/Cdc42 (135). Rac and Cdc42 are small ras-like, RhoA family GTPases, having an approximate molecular mass of 21 kD and are often referred to as p21 small G proteins. This family of GTPases is known to induce filopodia, lamellipodia and the disassembly of stress fibers, counteracting the action of RhoA which contributes to stress fiber assembly. Rac and Cdc42 are activated by guanine exchanger factors (GEF) such as vav, which contain PH domains, and thus are effectors for PI3K (2,84). This may explain why PI3K inhibition prevents cell-shape changes and detachment. Rac and Cdc42 activate several kinases including p21-activated kinases (PAK's) (139) which comprise a family of kinases that share homology in their kinase domains to yeast Ste-20 like kinases. Presently, at least three members have been cloned from mammalian cells. Interestingly, a dual role for PAK exists in that on the one hand it is involved in cytoskeletal reorganization and cell movement by phosphorylating myosin light chain kinase (31,132,138) and on the other hand, is able to mediate apoptosis via activation of JNK and p38 MAP kinases (25,92) (Fig. 1).

The anoikis/apoptosis signals:

The detached cells soon undergo apoptosis by a process referred to as anoikis (48). It has been speculated that anoikis involves MEKK and either one of the stress-activated kinases, JNK or p38MAPK (49), although an alternative mechanism might be responsible (70). The activation of JNK and p38MAPK by HRG, but not by EGF in LNCaP is consistent with this hypothesis. The apoptosis induced this way must be able to offset the anti-apoptotic effect of Akt activity, which is constitutively present in this cell type. p38MAPK activation is known to induce differentiation and apoptosis in several cell types, thereby providing additional support for this as a mechanism of cell death in this system (for examples, see (99,159,166)). Perhaps most germane to the present discussion is the report that in the breast cancer cell line SKBR3, HRG induces apoptosis via p38 activation and subsequent apoptosis (30).

Tyrosine Kinase Signals Through a Cytokine Receptor

IL-6 signals: hormone-independence, growth arrest, and neuroendocrine differentiation

Few interleukins are implicated in CaP progression; one that has drawn considerable attention is IL-6. Originally identified as a regulator of immune and inflammatory responses, IL-6 has now been recognized as a key factor involved in growth and metastasis of several types of neoplasms (62). IL-6 has a two-component receptor, the p80 Δ-subunit which binds IL-6 and the gp130 E-subunit which is the actual signal transducer and shared with other cytokine receptors such as oncostatin M (OM), leukemia inhibitory factor (LIF), and interleukin-11 (IL-11) (61). Both components of the IL-6 receptor are expressed in all prostate cancer specimens and cell lines surveyed, indicative of a role for IL-6 in CaP biology (141). However, this role is complex. In androgen-dependent cells such as LNCaP, dihydrotestosterone (DHT) suppresses IL-6 expression through a mechanism of androgen receptor-mediated repression of NF-NB activity (69). Addition of exogenous IL-6 to this cell line inhibits growth and induces neuroendocrine differentiation (37,116). By contrast, IL-6 functions as a growth factor for androgen-independent CaP cell lines, DU145 and PC3, increasing their growth rate (102), colony formation potential, and conferring resistance to certain chemotherapeutic agents and tumor necrosis factor (TNF)-mediated cell death (10,22,100) . The IL-6 autocrine loop is present in all androgen-independent CaP cells, but not in dependent lines (22) , suggesting that the IL-6 autocrine loop may be functionally linked to the androgen-independent phenotype. These observations, coupled with the finding that the level of circulating IL-6 is elevated in metastatic prostatic carcinoma (3,4) implicate IL-6 in CaP progression. While this view seems to be at odds with the antiproliferative effect of IL-6 in androgen-dependent cells such as LNCaP, it should be noted that neuroendocrine differentition (see below) results

in the release of neurotrophins which facilitate the survival and chemomigration of the surrounding CaP cells. The signal transduction pathways that give rise to the varied phenotypes are described below.

Hormone-independent growth and anti-apoptosis signals:

IL-6 is a strong inducer of growth and survival of androgen-independent DU145 and PC3 (11,12). Among the signals triggered by IL-6, MAPK is likely to be responsible for the growth response and PI3K for drug and apoptosis resistance, by analogy to the action of EGF. Since PC3 and DU145 do not express androgen receptors, the IL-6 induced growth and survival signals must pass through an androgen receptor-independent pathway. Interestingly, if the androgen receptor status is artificially restored by transfection into DU145, IL-6 is able to stimulate androgen receptor-dependent gene transcripton (*e.g.*, a reporter gene driven by the androgen response element (ARE)) in the absence of androgen (64). This could be inhibited by the nonsteroidal androgen receptor antagonist bicalutamide (Casodex), indicating that IL-6 indeed mediates its activation through the androgen receptor. The authors further showed that this activation requires the activities of PKC, PKA, and MAPK. In the AR-positive cell line LNCaP, IL6 is able to induce transcription from the prostate specific antigen (PSA) promoter in the absence of androgen (Li-Fen Lee , personal communication). Since PSA promoter activation critically depends on AR activity, the experiment described serves as confirmation of the ability of IL-6 to activate the androgen receptor. This is an intriguing finding which suggests that IL-6 may facilitate the initial transition of a prostate cancer cell from hormone-dependence to independence by acting as a pseudo-activator. Eventually, in the case of DU145 and PC3, androgen receptor expression is lost and the androgen receptor-independent IL-6 pathway takes over. Since AR is known to suppress the transcription of the IL-6 gene (69), the loss of AR further increases the expression of IL-6, setting up a permanent autocrine loop. Oncostatin M, which, like IL-6, activates gp130, is also capable of stimulating the growth of DU145 (12). In this case, STAT3 activation is required. This suggests that although diverse ligands interacting with similar receptors leads to an identical phenotype, diversification of signal transduction pathways contributes to CaP tumor progression and increases the likelihood for selection of aggressive phenotypes.

The growth arrest and neuroendocrine-differentiation signals :

In contrast to the robust growth stimulation of DU145 and PC3, IL-6 inhibits the growth of LNCaP and induces neuroendocrine (NE) differentiation (116). The acquisition of cells with NE characteristics has been reported to be an early marker for development of androgen-independence of prostate cancers and tumor cell populations have been reported to become enriched for NE cells following long-term anti-androgen therapy (23,41,101). It has been suggested that these NE cells function as a paracrine source of factors to support androgen-independent growth of the surrounding cancer cells. NE cells are identified by neurite outgrowth, the presence of neurosecretory granules, and by their ability to express a wide variety of neuronal-specific markers such as chromogranin A, neuro-specific enolase and a number of potentially mitogenic neuropeptide hormones including parathyroid hormone-related peptide, bombesin, serotonin, calcitonin, etc. (27). Increased serum levels of chromograinin A is found to correlate well with the acquisition of androgen–independence and CaP progression. Although NE cells are nonmitotic, proliferating carcinoma cells have been found in close proximity to them. These observations suggest that NE differentiation of prostate cells is associated with progression of CaP towards an androgen-independent state. The origin of NE cells in CaP is not entirely clear, although it has been suggested that they are derived from either prostate stem cells or prostate epithelial cells via transdifferentiation. The fact that some prostate cancer cell lines, such as LNCaP, can undergo NE differentiation suggests that at least a subset of NE cells is derived from prostate epithelial cells. In addition to IL-6 (116), a number of diverse stimuli have been described to induce NE phenotypes of prostate cancer cells. These include the cytokines IL-1 and IL-2 (42), long-term serum/androgen starvation (140), and elevation of intracellular cyclic AMP (cAMP) levels (27). A variety of physiological and pharmacological agents can increase cAMP levels, such as epinephrine, forskolin (adenylate cyclase activator), and the cAMP analogue dibutyryl cAMP (9,27). Interestingly, withdrawal of forskolin and epinephrine from LNCaP cells induce the loss of neuritic processes and re-acquisition of a morphology typical of untreated cells indicating that NE differentiation is reversible and that the affected cells were arrested in growth, but not terminally differentiated and senescent. In the IL-6 induced differentiation model system, treated LNCaP cells were shown to be growth-arrested at the G1/S boundary (93,116). This growth arrest apparently involves the activation of p27 at the transcriptional level, but is independent of SHP2 association with IL-6R (73,93). Taken together, since it has been demonstrated that elevated cAMP levels can potentiate IL-6 signal transduction, cross-talk between the cAMP and IL-6 pathways might be further augment NE differentiation (20). An even more dynamic and aggressive environment can conceivably be established after malignant prostatic neuroendocrine begin secreting mitogenic neuropeptides which can also contribute to cAMP elevation.

What are the signal pathways leading to neuroendocrine differentiation of LNCaP cells? The studies of IL-6 (116) and cAMP agonists (27) have provided insight into this process. In the case of cAMP elevation, it is expected that PKA is involved, and indeed forced expression of a dominant-negative mutant of PKA blocks the differentiation process (M. Cox and S. Parsons, personal communication). PKA activates CREB and ATF and these transcriptional factors are likely to be responsible for activating the genes involved in neuroendocrine differentiation. On the other hand, in LNCaP, PKA also activates the MAPK pathway via Rap1, which leads to jun/fos activation (19). Thus, a combination of these bZIP proteins may be involved in this phenotype. The mechanism whereby IL-6 induces NE was more obscure. Early studies in PC12, a rat pheochromocytoma cell line which can be induced by IL-6 to undergo neuronal differentiation, revealed the role of the PI3K pathway in this process (90,143,158,163). When LNCaP was treated with IL-6, the MAPK, JAK/STAT3 and PI3K pathways were all strongly activated (116) (Fig. 2). In addition, a PH-domain containing tyrosine kinase, Etk/Bmx was also activated and serves as an effector molecule for PI3K. This is understandable, as PH-domain interacts with 3'-phosphoinositides, metabolic products of PI3K (131). By analogy to Btk and Itk, close cousins of Etk, 3'-phosphoinositide binding unfolds this family of proteins, exposing their kinase domains and translocating them to the cytoplasmic membrane, where they can be activated by src-like kinases (5). That Etk/Bmx is crucial for IL-6 induced neuroendocrine differentiation was demonstrated by the differention-resistant phenotype acquired by LNCaP cells stably tranfected with a dominant-negative Etk expression construct (116). A key question was then what transmits the high level of tyrosine phosphorylation signal, considering the fact that IL-6 receptor itself is not a tyrosine kinase. Jak family kinases known to be activated by cytokine are possible candidates. Tyk2 is indeed activated in IL-6 treated LNCaP cells, but more intriguingly, ErbB2 and ErbB3 are also activated (115) (Fig. 2). ErbB1, the only other receptor tyrosine kinase in this family expressed in LNCaP cells, is not activated, indicating the specificity of this cross talk. Furthermore, ErbB2 forms a stable complex with the gp130 subunit of the IL-6 receptor in an IL-6 dependent manner. This suggests that IL-6 activation of ErbB kinases is through direct engagement and that ErbB2 and Tyk2 both contribute to the elevation of tyrosine phosphorylation induced by IL-6 in LNCaP. Using the ErB2-trapping approach described above (*i.e.* single-chain antibody expression construct) to functionally knock out ErbB2, it was shown that both ErbB3 and MAPK activation require ErbB2 engagement. This example illustrates how a cytokine receptor can diversify its signal by engagement with receptor tyrosine kinases, which may help explain the complex and pleitropic phenotypes induced by IL-6. Consistent with a role for ErbB2 in neuroendocrine differentiation is the demonstration that prostatic neuroendocine cells with dendritic appearance and chromogranin A expresson express ErbB2 (67). From the above discussion, it appears that at least the PKA and PI3K/Etk pathways are involved in the neuroendocrine differentiation of LNCaP. Most likely, other pathways such as MAPK or STAT3 also contribute to this phenotype.

Summary

Tyrosine kinases play significant roles in cellular signaling in prostate cancer. They respond to not only growth factors, but also to cytokines. The widely recognized pathways such as MAPK, PI3K, PLC-γ and STATs are activated by multiple inducers and they obviously participate in the signaling (Fig. 1). Yet, it is clear they are not the whole story and the final outcome depends not only on the combination of multiple pathways, but also on their relative intensities as well. By design, we have focused on signals mediated by ErbB kinases. Fig.2 gives a summary of the different signal pathways outlined in this article. Whereas TGF-Δ/EGF is a strong growth stimulator for all CaP cells so far studied, HRG induces anoikis and apoptosis. These two diametrically opposite phenotypes nevertheless share overlapping signals. For instance, both TGF-Δ and HRG activate SHC/MAPK strongly (thick arrows) and PI3K to different extents. By contrast, PLC-γ is solely induced by TGF-Δ and p38MAPK by HRG. The latter signals thus may be responsible for the particular phenotypes

induced by individual growth factors. A comparison of HRG and IL-6 signal pathways offers another intriguing scenario. Here, both involve an ErbB2/ErbB3 complex, one activated by HRG from outside and the other activated by complex formation with IL-6 receptor from within. Again, common signals associated with ErbB2 and ErbB3 such as MAPK and PI3K are induced. Yet, the biological consequences are profoundly different. The likelihood that Tyk2/STAT pathway contributes to the unique neuroendocrine phenotype needs to be studied. How and whether it is related to cAMP agonist induced neuroendocrine phenotype are also worth exploring. While complicated, prostate cancer offers a very interesting biological system to dissect key signal molecules involved in cell-fate determination. The understanding of these pathways is not merely a scientific curiosity, but may benefit strategies to modify the tumor behavior (*e.g.* to make them more prone to apoptosis and enhance sensitivity to therapy). The success of Herceptin may herald the use of anti-tyrosine kinase antibodies or inhibitors as general anti-cancer agents. In addition, future intervention strategies might include potentiating the toxicity of Herceptin by combination with heregulin, and also disruption of autocrine growth factor/receptor loops. In light of recent findings, it will also be critical to consider the interplay of androgen receptor signaling with the pathways discussed in the design of tyrosine kinase-based therapies. For the above reasons, coupled with the possibility of uncovering potential tumor markers, the study of tyrosine kinases and cellular signaling in prostate cancer promises to be a thriving area of research in the next millennium.

Acknowledgements

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Table 1. Tyrosine kinase profile in CWR22 CaP xenograft

Receptor TK		Non-Receptor TK	
family	**members**	**family**	**members**
EGFR	ErbB1	*Src*	src
	ErbB2		yes
	ErbB3		lck
Eph	EphA1	*CSK*	Csk
	EphA2		
	EphA4	*Src-B*	Frk
	EphB4		
		JAK	JAK1
UFO/Ax1	Sky/Tyro3		tyk2
	Nyk/mer		
		Abl	abl
Ddr	Ddr1		arg
	Ddr2		
		Btk	Etk/Bmx
PDGFR	PDGFR		
		FAK	FAK
FGFR	FGFR2		
	FGFR4	*ZAP70*	Syk
InR	IGFR		
MET	MET		
	Ron		
RET	RET		
NGFR	trkA		
	trkC		

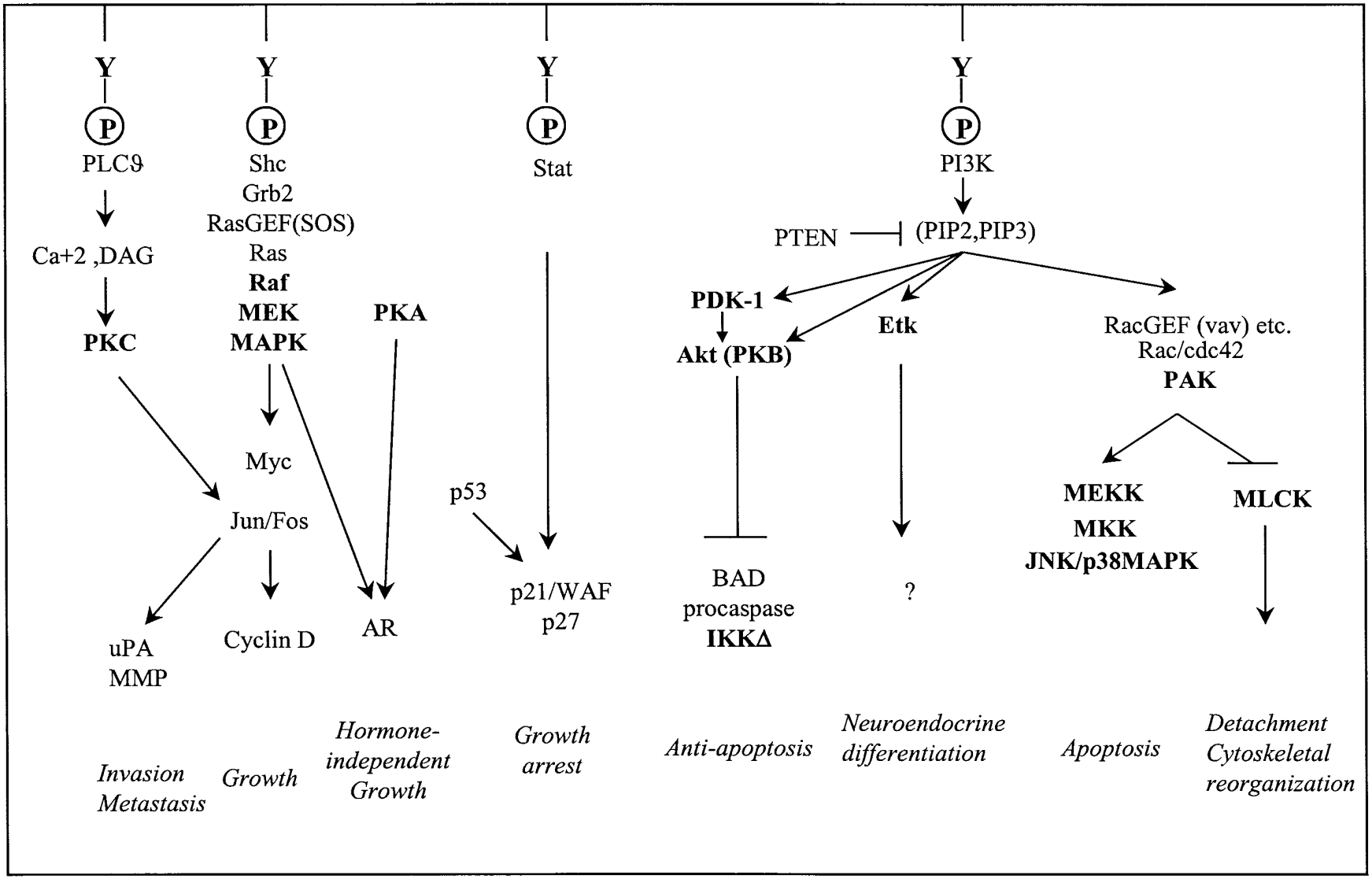

Figure 1.: Summary of the putative signal transduction pathways of prostate cancer cells initiated by tyrosine phosphorylations. Arrows indicates activation of down stream substrates. T-shaped bars indicate inactivations. The highlighted molecules are tyrosine or seirine kinases. The nomenclatures are described in the text. The data is baased primarily on the studies of erbB family of kinases.

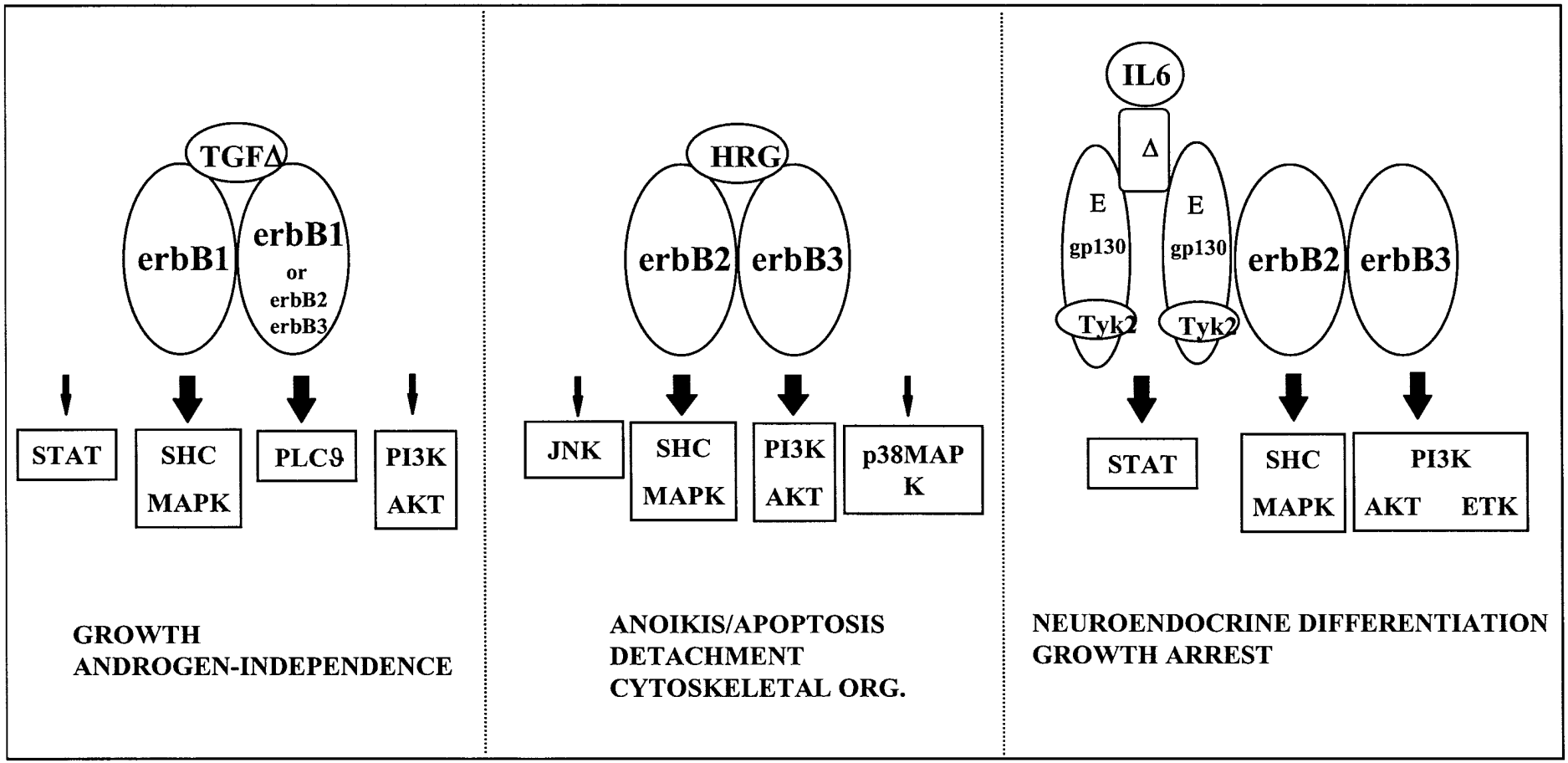

Figure 2.: Schematic representation of key ErbB-mediated signal transduction pathways induced by TGF-Δ, HRG, and IL-6 in LNCaP prostate cancer cells. Signaling is initiated by ErbB receptor heterodimerization induced by direct ligand binding (*i.e.* TGF-Δ, HRG) or indirectly via association with the activated IL-6 receptor. Activation of the intrinsic ErbB tyrosine kinase activity leads to tyrosine phosphorylation of intracytoplasmic domains, recruitment of proteins containing SH2 or PTB domains, and propagation of the signal. Well-characterized signaling pathways in this system are annotated in boxes and the relative strengths of each pathway are indicated by the thickness of the arrows.